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Abbreviations:

ED: Endocrine Disruptor

GD: Gestational Day

NMR: Nuclear Magnetic Resonance

OSC: Orthogonal Signal Correction

PCA: Principal Components Analysis

PLS-DA: Partial Least Squares-Discriminant Analysis

PND: Post-Natal Day

VIP: Variable Importance in the Projection

Abstract

BACKGROUND: Bisphenol A (BPA) is a well-known endocrine disruptor used to manufacture polycarbonate plastics and epoxy resins. Exposure of pregnant rodents to low doses of BPA results in pleiotropic effects in their offspring. Metabolomics, a method used to reveal metabolic changes in response to nutritional, pharmacological, or toxic stimuli is used here to examine effects of BPA exposure.

OBJECTIVES: We report the use of metabolomics to identify metabolic shifts induced *in vivo* by perinatal exposure to low doses of BPA in CD-1 mice.

METHODS: Male offspring born to pregnant CD-1 mice that were exposed to vehicle, 0.025µg, 0.25µg or 25µg BPA/kg BW/day, from gestational day 8 through day 16 of lactation, were examined on PND2 or PND 21. Aqueous extracts of newborns (PND2, whole animal), and of livers, brains, and serum samples from PND21 pups were submitted to ^{1}H NMR spectroscopy. Data were analyzed using Partial Least Squares Discriminant Analysis (PLS-DA).

RESULTS: Examination of endogenous metabolic fingerprints revealed remarkable discrimination in the four PND2 newborn groups (whole extracts) strongly suggesting changes in the global metabolism. Furthermore, at PND 21, statistical analyses also successfully discriminated among treatment groups for liver, serum, and brain samples. Variations in glucose, pyruvate, some amino-acids, and neurotransmitters (GABA and Glutamate) were identified.

CONCLUSIONS: Low doses of BPA disrupt global metabolism, including energy metabolism and brain function in perinatally exposed CD-1 mouse pups. Metabolomics can be used to highlight the effects of low doses of endocrine disruptors by linking perinatal exposure to changes in global metabolism.

Introduction

Exposure to bisphenol A (BPA), which is manufactured at a rate of $>3.10^6$ tons/year, is ubiquitous. Its possible impact on human health is reflected in recent worldwide regulatory legislation. The Canadian authorities followed by the European Union have recently banned the use of BPA in infant feeding bottles, a landmark move to safeguard the health of infants and the general population (Asimakopoulos et al. 2012; European Commission 2011). Nevertheless, BPA is still widely used in polycarbonates, epoxy resins, paints, lacquers, and medical devices. BPA is also used as a base compound for the manufacture of flame-retardants, brake fluids, and thermal papers (Fernandez et al. 2007). BPA monomers migrate out of these products and contaminate food, beverages, intravenous infusions, etc (Goodson et al. 2004). Although the main route of contamination is through ingestion, the trans-dermal route could also contribute to BPA exposure in humans when direct contact with BPA (free monomer) occurs (Zalko et al. 2011). Detectable levels of BPA were present in urine samples of over 92% of Americans tested by the Centers for Disease Control in 2003 (Calafat et al. 2008). Higher exposure levels were found in children and adolescents when compared with adults. Of particular concern is the finding of high levels of BPA in premature infants being treated in neonatal intensive care units (Calafat et al. 2009). BPA has also been detected in maternal and fetal plasma, in human placenta, and in the milk of nursing mothers (Calafat et al. 2006; Sun et al. 2004). Animal studies have highlighted the estrogenic effects of BPA (vom Saal et al. 2007) although BPA is considered by some to be a weak estrogen due to its low potency when compared to estradiol in reporter gene assays involving nuclear receptors (Blair et al. 2000). However, recent work has shown that BPA can be as potent or more potent than estradiol in promoting some estrogenic activities (Alonso-Magdalena et al. 2006; Alonso-Magdalena et al. 2012). BPA can also bind to

membrane receptors (GPR30, and the membrane form of estrogen receptor-alpha), to produce similar effects to those of estradiol (Thomas and Dong 2006; Welshons et al. 2006; Wozniak et al. 2005).

Perinatal BPA exposure decreased fertility and fecundity in female CD-1 mice (Cabaton et al. 2011), and decreased fertility in male offspring of exposed rats (Salian et al. 2011). Additional effects of perinatal BPA exposure include masculinization of behaviors and brain structures in female CD-1 mice. Exposure to BPA through placenta and milk has been shown to increase early adipose storage and adipogenesis in a sex-specific and dose-dependent manner in rats and mice, with consequences on body weight later in life (Rubin and Soto 2009; Somm et al. 2009). BPA exposure has been linked to altered glucose homeostasis in pregnant female rodents and their male offspring (Alonso-Magdalena et al. 2010) and has been postulated to be a contributing factor in predisposing populations to the development of obesity and diabetes later in life (Heindel and vom Saal 2009; vom Saal et al. 2012).

Metabolomics aim at measuring the global, dynamic metabolic response of living systems to biological stimuli (Nicholson and Lindon 2008). Metabolomics have furthered our understanding of drug toxicology, while complementing more traditional approaches (Coen 2010). The integration of metabolomics and conventional toxicological studies is expected to provide valuable information for risk assessment of endocrine disruptors (EDs) such as BPA. Metabolic fingerprints based on nuclear magnetic resonance (NMR) spectroscopy, combined with appropriate statistical methods, could detect slight changes in the metabolome of cells, tissues or organisms exposed to EDs, opening the way to examine whether exposure to an ED results in global alterations of metabolism, and whether these changes persist after cessation of exposure.

Metabolomic data are characterized by a large number of variables and by high correlations among these variables. Multivariate methods, such as PCA and PLS-DA, which aim at solving both problems mentioned above, are then the methods of choice to analyze metabolomic data. The aim of PCA is to explain the variation in the dataset, regardless of the origin of this variation. PLS-DA is an alternative to PCA that allows discriminating observations according to classes defined *a priori*. Besides, PLS-DA is preferred to PCA for sample discrimination because the dimension reduction provided by PLS is explicitly guided by inter groups ("among groups") variability, whereas PCA can only identify gross variability directions and is incapable of distinguishing variability that occurs ''among-groups'' and ''within-groups'' (Quintás et al. 2012). When the number of groups is greater than 2, PLS-DA is more appropriate than PCA (Lindon et al. 2004).

We hypothesized that the global metabolism of mice perinatally exposed to BPA may be disrupted. The present study was designed to test this hypothesis in the outbred CD-1 mouse, a common animal model in BPA studies. Rodent strains vary in their susceptibility to low-dose BPA exposure (Richter et al. 2007). Rodent placentation at late stages of gestation is histologically close to human placentation (Zalko et al. 2003), and the mouse and rat have been shown to be excellent models for understanding the human diethylstilbestrol syndrome observed in offspring of mothers exposed during pregnancy (Vandenberg et al. 2009). Previous work using CD-1 mice has shown that, following trans-placental transfer, fetuses become exposed to their parent's BPA burden as well as BPA metabolites (Markey et al. 2001; Wadia et al. 2007; Zalko et al. 2003). The body of literature clearly demonstrates multiple targets affected by low doses of BPA, including metabolism, especially when the exposure occurs at critical periods of development (Vandenberg et al. 2012). In the present study, CD-1 fetuses and neonates were

exposed to low doses of BPA (0.025µg, 0.25µg or 25µg BPA/kg BW/day) administered to their mothers from the end of gestational day (GD) 8 through day 16 of lactation. Female offspring were examined for reproductive outcomes and found to have decreased fertility and fecundity (Cabaton et al. 2011) while the male offspring were examined for changes in global metabolism by assessing ^{1}HNMR profiling and analyzing the data using multivariate statistics. Male offspring were examined at PND2 (whole body), and at PND21. For the latter group, samples included the liver (the main metabolizing organ), the serum (circulating metabolites), and the brain, given the fact that recent studies have suggested that perinatal exposure to low doses of BPA could have persistent effects on the brain structure, function and behavior in rodents (Richter et al. 2007).

Materials and Methods

Chemicals.

Dimethyl-sulfoxide (DMSO, CAS #67-68-5) and bisphenol A (4,4'-dihydroxydiphenyl-dimethylmethane, CAS #80-05-7, product #239658, lot #03105ES; purity ≥99%) and butylated hydroxytoluene (BHT) were purchased from Sigma Chemical Company (Saint Louis, MO). BPA stock purity was confirmed as described previously (Cabaton et al. 2011). Acetonitrile was purchased from Scharlab SL (Sentmenat, Spain), deuterium oxide (D_2O) and sodium 3-trimethylsilyl-2,2,3,3-tetradeuteriopropionate (TMSP) from Euriso-top (Saint-Aubin, France).

Animals.

Twelve week old female CD-1 mice, and proven breeder male mice (Charles River Laboratories, Wilmington, MA) were maintained in temperature and light controlled (14/10-h light/dark cycle)

conditions at the Human Nutrition and Research Nutrition Center AAALAC-approved animal facility in Boston, MA. All experimental procedures were approved by the Tufts University-Tufts Medical Center Institutional Animal Care and Use Committee. The animals were treated humanely and with regard for alleviation of suffering. Cages, water, and bedding all tested negligible for estrogenicity by the E-SCREEN assay (Soto et al. 1992). Water (glass bottles) and food (Harlan Teklad 2018; Indianapolis, IN) were supplied *ad libitum.* The estrogenicity of food lots was measured by the E-SCREEN assay and was also tested independently by Dr Welshons (Welshons et al. 1990), confirming a negligible estrogenic activity (less than 20pmol of estrogen equivalents per gram).

Mice were allowed to acclimatize for 5 days before being paired to mate. The morning on which a vaginal plug was detected was considered GD 1. On the evening of GD8, dams were implanted subcutaneously with Alzet osmotic pumps (model 2004, Alza Corp, Palo Alto, CA) following the manufacturer recommendations. Pumps were designed to deliver vehicle alone (50% DMSO in water), or 0.025µg (BPA 0.025µg/kg), 0.25µg (BPA 0.25µg/kg) or 25µg (BPA 25µg/kg) BPA/kg body weight (BW)/day. These pumps continued to release at a constant rate (0.25µL/h) until day 16 of lactation. The actual delivered dose of BPA decreased as pregnancy progressed because the BPA dose was calculated based on the weight of the mother at GD6, and body weights increased from this point throughout pregnancy. More than 90% of the dams delivered naturally and the F1 litters were culled to 8 pups, with no sex ratio differences between groups on the day after birth. Litters were weaned on PND 21.

Experimental design.

At PND2, one F1 male mouse that had either been exposed perinatally to vehicle or BPA was randomly chosen from each litter and euthanized by decapitation (control: N=20; BPA 0.025µg/kg: N=18; BPA 0.25µg/kg: N=14; BPA 25µg/kg: N=11). At PND 21, one F1 exposed male mouse was randomly chosen from each litter and euthanized (CO_2 euthanasia). Blood (control: N=11; BPA 0.025µg/kg: N=12; BPA 0.25µg/kg: N=14; BPA 25µg/kg: N=12), brain and liver (control: N=11; BPA 0.025µg/kg: N=11; BPA 0.25µg/kg: N=13; BPA 25µg/kg: N=14) were collected.

Sample preparation for ^{1}H NMR spectroscopy.

Trunk blood was collected in serum tubes (Sarstedt, Newton, NC, USA). Blood samples were centrifuged for 5min at 10,000g and 20°C. Serum was collected in microtubes and stored at -20°C. Serum samples (100µL) were diluted with 600µL of D_2O and centrifuged at 5,000g for 10min before they were placed in 5mm NMR tubes.

For liver, brain and whole pup samples, extraction procedures were derived from Folch et al. (1951) and from the method described by Waters et al. (2002). Samples of tissue (liver: 100mg; whole brain; whole pup) were homogenized using a Polytron PT2100 in acetonitrile/H_2O (50/50, v/v) containing 0.1% BHT in an ice-water bath. Homogenates were centrifuged at 5,000g for 10min at 4°C and the supernatants were removed and lyophilized. The lyophilisates were reconstituted in 600µL of D_2O containing 0.25mM TMSP (as a chemical shift reference at 0ppm). The reconstituted solutions were transferred to NMR tubes.

^{1}H Nuclear Magnetic Resonance (NMR) analyses.

All ^{1}H NMR spectra were obtained on a Bruker DRX-600-Avance NMR spectrometer operating at 600.13 MHz for ^{1}H resonance frequency using an inverse detection 5mm ^{1}H-^{13}C-^{15}N cryoprobe attached to a CryoPlatform (the preamplifier cooling unit).

The ^{1}H NMR spectra were acquired at 300K using the Carr-Purcell-Meiboom-Gill (CPMG) spin-echo pulse sequence with pre-saturation, with a total spin-echo delay ($2n\tau$) of 100ms to attenuate broad signals from proteins and lipoproteins. A total of 128 transients were collected into 32k data points using a spectral width of 12ppm, a relaxation delay of 2.5s and an acquisition time of 2.28s. Prior to Fourier Transformation, an exponential line broadening function of 0.3 Hz was applied to the FID.

To confirm the chemical structure of metabolites of interest, 2D ^{1}H-^{1}H COSY (Correlation Spectroscopy) and 2D ^{1}H-^{13}C-HSQC (Heteronuclear Single Quantum Coherence Spectroscopy) NMR experiments were performed on selected samples.

Data reduction and multivariate statistical analyses.

All NMR spectra were phased and baseline corrected, then data was reduced using AMIX (version 3.8, Bruker, Analytik) to integrate 0.01 ppm wide regions corresponding to the δ 10.0-0.5 ppm region. The δ 5.1-4.5 ppm region, which includes the water resonance, was excluded. A total of 791 to 861 NMR buckets were included in the data matrices. To account for differences in sample amount, each integrated region was normalized to the total spectral area. Multivariate analyses were used to study the effect of the treatment (DMSO/0.025µg, 0.25µg or 25µg BPA/kg BW/day) on the metabolome. Principal components analysis (PCA) was first performed to reveal

intrinsic treatment-related clusters and detect eventual outliers. Partial least squares–discriminant analysis (PLS-DA) was then used to model the relationship between group and spectral data. PLS-DA is similar to PCA but uses discriminant variables that correlate to class membership. Before analysis, orthogonal signal correction (OSC) filtering (Wold et al. 1998) was used to remove variation not linked to the treatment (physiological, experimental or instrumental variation). Filtered data were mean-centered and scaled (Unit or Pareto). For all the figures, Hotelling's T^2 statistics were used to construct 95% confidence ellipses. The R^2Y parameter represents the explained variance. Seven-fold cross validation was used to determine the number of latent variables to include in the PLS-DA model and to estimate the predictive ability (Q^2 parameter) of the adjusted model. In addition, the robustness and validity of the PLS-DA results were calculated using a permutation test (number of permutations= 200). Discriminant variables were determined using VIP (Variable Importance in the Projection), an appropriate quantitative statistical parameter ranking the descriptors according to their ability to discriminate different doses. This global measure of the influence of each variable on the PLS components was used to derive a subset of the most important metabolites for the separation of experimental groups. Then Kruskal-Wallis test was used to determine metabolites significantly different between the groups. SIMCA-P software (V12, Umetrics AB, Umea, Sweden) was used to perform the multivariate analyses.

Results

No statistical difference in the weight of PND2 body pups, PND21 livers or brains was observed. In a first step, PCA was applied to the NMR spectral data sets of BPA exposed PND2 whole body pups aqueous extracts, as well as serum, liver and brain extracts from PND21 male

(Supplemental Material, Figures S1-S4). PCA score plots allowed a primary separation between groups. Then, a supervised PLS-DA model was built to further investigate the differences between groups.

PND 2 whole body pups aqueous extract.

For the 4 experimental groups taken together, the analysis generated a 3 latent components PLS-DA model characterized by a faithful representation of the data (R^2Y=71.5%), and, more importantly, by a good cumulative predictive capacity (Q^2=0.557) (Figure 1A). The score-plot of the PLS-DA showed a clear separation between the Control group and the BPA groups; 83 variables had a VIP value> 1.0 (arbitrary threshold) and the median of 71 buckets were statistically different according to the Kruskal-Wallis test. These differences were later found to correspond to 20 metabolites, according to 2 dimensions NMR spectra identifications. Endogenous metabolite variations induced by BPA exposure (25µg BPA samples compared to control samples) in PND2 pups showed an increase in lactate, glucose, cholines, creatine, and glycine. Conversely, a decrease in valine, leucine, isoleucine and lysine was observed (Table 1). In addition, taken separately and using a pairwise comparison, all groups could be successfully discriminated , including the BPA0.025µg and BPA0.25µg groups (Table 2; Figure 1B). The latter analysis generated a 3 latent components PLS-DA model characterized by a very faithful representation of the data (R^2Y=98.0%) with more that 90% variability explained along axis 1, and by a very good cumulative predictive capacity (Q^2=0.731). This analysis identified 13 metabolites contributing to the difference in metabolic profiles between BPA0.025µg and BPA0.25µg groups by VIP (>1) and Kruskal-Wallis test (Table 1).

PND 21 serum samples.

The score-plot of the PLS-DA using the entire dataset (all groups taken together) showed a clear separation between the BPA25µg group and all other groups, along the first latent component, and between the BPA0.25g and BPA0.025µg groups (Figure 2A). This analysis generated a 2 latent components model with R^2Y=55.3% and Q^2=0.450. Finally, 13 metabolites were identified as discriminant parameters in the metabolic profiles. Further pairwise comparisons between Control and BPA0.025µg samples, on one hand, and between control and BPA0.25µg samples, on the other hand, demonstrated a clear discrimination between the corresponding groups and 14 and 11 metabolites were identified as discriminant biomarkers respectively (Figure 2B and 2C, respectively). BPA exposure (BPA25µg samples compared to Control samples) induced a decrease in lipids (LDL/VLDL) and lactate concentrations and an increase in glucose, taurine and cholines (Table 1).

PND 21 liver extract.

The 4-group comparison generated a 2 latent components PLS-DA model characterized by a correct representation of the data (R^2Y=48.3%) and Q^2 equal to 0.421. The score-plot of the PLS-DA showed a clear separation between Control, BPA0.25µg, and BPA25µg, as well as BPA0.25µg and BPA25µg (Figure 3A). The median of 46 buckets were significantly different according to the Kruskal-Wallis test, corresponding to 3 metabolites discriminating the 3 BPA-exposed groups, 8 metabolites discriminating between BPA0.25µg and BPA25µg and 5 metabolites separating Control and BPA0.025µg from BPA0.25µg and BPA25µg (Table 2). For more specificity, pairwise comparisons were carried out between the BPA0.025µg group and all other groups, which led to the separations displayed in Figure 3B, 3C and 3D, and further

detailed in Table 1. When comparing 25µg BPA samples with Control samples, endogenous metabolite variations induced by BPA exposure showed an increase of taurine, glutamate and glutathione. A decrease in lactate, glucose, cholines and glycogen was also observed (Table 1).

PND 21 brain extract.

The 4-group comparison generated a 3 latent components PLS-DA model characterized by a faithful representation of the data (R^2Y=78.9%) and a good cumulative confidence criterion of prediction (Q^2=0.564). The score-plot of the PLS-DA showed a correct separation between Control animals and all BPA groups (Figure 4); 101 variables had a VIP value > 1.5 and the median of 76 buckets were statistically different according to the Kruskal-Wallis test, corresponding to 21 metabolites. Taken separately using pairwise comparisons, all groups could be discriminated from each other (Table 2). Endogenous metabolite variations between 25µg BPA and Control samples showed an increase in glutamine, glycine and aspartic acid concentration, and a decrease in cholines, glutamate and γ-aminobutyric acid (GABA) (Table 1).

Discussion

Developmental exposure to BPA affects the reproductive system and fertility, alters brain development and behavior, disrupts glucose homeostasis, and may contribute to the development of obesity and metabolic syndrome (Cabaton et al. 2011; Richter et al. 2007, Alonso-Magdalena, 2010; Ryan et al., 2010). In the present study, we examined whether a novel approach based on metabolomics profiling could detect subtle changes in the metabolome following BPA exposure at low to very low doses, and whether metabolic profiling could reveal differences that persist beyond the end of the exposure period. The BPA doses used in this study correspond to 1/2,000

to 1/2,000,000 of the no observed adverse effect level (NOAEL) for BPA (EFSA 2006; FDA 2008), and the time of BPA exposure coincided with critical periods of development (GD8 to PND16).

Factors that impact fetal growth are also associated with postnatal growth rate and adult body weight in humans as well as laboratory animals (Barker 2004; Coe et al. 2008). The classical tools of toxicology are not designed to detect the effects of an early exposure to low doses of endocrine disruptors. Recent developments in metabolomics allow to further explore global changes in biological systems. NMR spectroscopy is broadly used in research to characterize metabolite structure. NMR spectroscopy fingerprints combined with multivariate statistical analysis provides a powerful tool to detect metabolic changes induced by very low doses of EDs allowing discrimination between several experimental groups, based on over- or under-expression of endogenous molecules. In the current study, we used ^{1}H-NMR, followed by PCA and PLS-DA analyses. PCA was used for the detection of potential outliers. PCA does usually allow only a first-step discrimination between groups. When the number of groups is greater than 2, PLS-DA is more appropriate (Lindon et al. 2004). We performed PLS-DA to explore NMR fingerprints linked with BPA perinatal exposure, PLS-DA being now routinely used in the field of metabolomics. Linear combinations of NMR buckets were constructed and were used to visualize differences (or similarities) between groups. One primary objective of our study was to find exposure biomarkers that would correlate with the metabolic changes triggered by perinatal exposure to BPA, thus providing a proof of concept that ^{1}H-NMR, completed by robust statistical analysis, is a powerful tool that would discriminate between exposed versus non-exposed animals.

PLS-DA is a widely used classification method in metabolomics to discriminate different treatment groups and identify biomarkers responsible for this discrimination (Gavaghan et al. 2002; Martin et al. 2009). Our models were cross-validated and their robustness was assessed with permutation tests. A robust model is associated with a Q^2 value greater than 0.40 (McCombie et al. 2009). This was the case for all our models. The parameters of the PLS-DA models were estimated based on datasets comprising 49 (PDN21) to 63 (PND2) individuals. Since a large number of variables had to be processed, cross-validation was necessary to avoid over-fitting. Results of permutation tests demonstrated that our analyses were robust and not obtained by chance. Discriminating variables were identified using the VIP and Kruskal-Wallis test, providing variables that explain the discrimination between groups. The next step was to connect some discriminant metabolites with possible mechanisms of action, and to suggest some exposure biomarkers.

We were able to separate groups of pups that were perinatally exposed to low doses of BPA, (0.025µg/kg BW/d), with robust statistical models (Q_2>0.4) even at a very early stage of life (PND2). Among the discriminating variables, glucose was affected by BPA perinatal exposure. Alonso-Magdalena et al. (2010) have also shown that BPA exposure during pregnancy disrupts glucose homeostasis in 6-month male offspring. It makes sense that the shift observed for glucose would in turn be involved in the disruption of pyruvate biosynthesis through glycolysis. Pyruvate supplies energy to living cells through the Krebs cycle (aerobic respiration), and alternatively, ferments to produce lactate (anaerobic respiration); pyruvate was also found to increase in PND2 pups. In this case, lactate could still be utilized by neurons as already demonstrated in mice, rats, and humans (Wyss et al. 2011; Zilberter et al. 2010). At PND2, two other variables (increased levels of creatine and glycine) contributed to the discrimination

between the control and BPA25µg groups, respectively. Creatine, a product of amino acid degradation (including glycine), is a major metabolite mainly found in muscle and brain and appears to be affected by prenatal BPA exposure. An increase in creatine levels might lead to an increase in ATP production, and consequently to increased energy metabolism. Glycine, a key precursor of porphyrins involved in heme production, is an inhibitory neurotransmitter in the central nervous system, especially in the spinal cord and in brainstem, as well as in the retina. An increase in glycine may disrupt the global energy metabolism as well as brain and neurologic functions. Conversely, a decrease in essential amino-acids, namely valine, leucine and isoleucine could reflect a disruption in their degradation pathways, which would be consistent with previous observations in GD18 rat fetuses exposed to butylbenzylphthalate (Sumner et al. 2009). Finally, we found that BPA exposure increased cholines concentrations in PND2 mice, which may impact membrane integrity and could favor a decrease in acetylcholine production in the brain.

In PND21 tissues, our data were able to discriminate all BPA exposed groups from controls. As already observed in PND2 mice, glucose in serum and liver was affected by perinatal BPA exposure. Similarly, cholines were also decreased reflecting a potential disruption in the membrane integrity. The decrease in lipids (VLDL-HDL) in PND21 serum samples, as well as an increase in taurine (involved in the conjugation of bile acids) suggest that lipid metabolism may also be affected by a BPA exposure in PND21 mice. Taurine is a key amino-acid for cardiovascular function, as well as for the development and function of skeletal muscle. Taurine, a major constituent of bile, was decreased in liver (and serum) in PND21 mice, reflecting a possible disruption in the digestive process. In liver extracts, an increase in glutathione in animals exposed to 25µg BPA/kg BW/d suggested a possible modulation of this detoxification

pathway, and also a hyper-production of pyruvate, a key metabolite involved in the Krebs cycle, glycolysis and glycogenesis. These changes may again affect energy metabolism pathways.

Regarding PND21 brain extracts, recent papers have highlighted brain development alterations following BPA perinatal exposure (Itoh et al. 2012; Kunz et al. 2011). In our study, 2 neurotransmitters, i.e. γ-amino-butyric acid (GABA) and glutamate, were significantly decreased in all BPA exposed animals, compared to controls. Glutamate, the major excitatory transmitter in the brain, is the precursor for the synthesis of GABA, the major inhibitory transmitter in the adult brain. It is important to note that prior to their neurotransmission roles in adulthood, both glutamate and GABA are thought to influence processes of neural development including proliferation, migration, differentiation, and survival (Lujan et al. 2005). Therefore decreased levels of these neurotransmitters during postnatal development could be expected to exert lasting effects on the brain. In our study, glutamine (the precursor of glutamate) was found to be increased in the brain, whereas glutamate levels decreased, suggesting a conversion problem, and possibly brain function disruption and/or damage. Aspartate, produced from oxaloacetate (by transamination) is another neurotransmitter that stimulates *N*-methyl-D-aspartate receptors, the predominant molecule that controls synaptic plasticity and memory function. An increase in aspartate, known as an excitatory neurotransmitter, which increases the likelihood of depolarization in the postsynaptic membrane, might induce behavioral hyperactivity (Ishido et al. 2011).

In summary, we have observed significant and unequivocal shifts in the global metabolism of young males perinatally exposed to low doses of BPA. Energy metabolism and brain function are the main targets suggested by metabolomics studies on extracts from PND2 and PND21 males. Further work is ongoing on samples from female mice perinatally exposed to the same

doses of BPA, euthanized at later time-points with the aim to better understand the mechanisms of action of BPA and the lasting effects of BPA long after the time of exposure.

Regarding BPA, this study provides a proof of concept that metabolomics, here as NMR-based metabolic fingerprints, can be used as a novel approach and powerful tool to detect metabolic shifts following perinatal exposure to BPA, even at very low doses. A similar approach could be used for other EDs, and is expected to contribute to a better understanding of the modulations/disruption of metabolome triggered during critical windows of development. Metabolomics could also be suitable and reliable for the analysis of biological fluids such as urine or blood in animal models, as well as human populations.

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Table 1. Endogenous metabolite variations induced by BPA exposure (25µg BPA samples compared to control samples) in PND2 pups, and in serum, liver and brain of PND21 mice.

Metabolites	^{1}H NMR Chemical shifts δ(ppm)	Whole body PND2	Serum PND21	Liver PND21	Brain PND21
Lipids[a]	0.86(m); 0.90(m);1.26(m); 1.30(m)		-		
Lactate	1.33(d); 4.11(q)	+	-	-	
Glucose	3.54(m);3.66(m);3.70(m); 3.74(m);3.78(m);3.82(m); 3.86(m);3.90(m)	+	+	-	
Taurine	3.26(t);3.42(t)		+	+	
Cholines[b]	3.20(s);3.22(s);3.23(s)	+	+	-	-
Creatine	3.03(s);3.93(s)	+			
Glutamate	2.08(m);2.34(m)			+	-
Glutamine	2.14(m);2.46(m)				+
Glycine	3.55(s)	+			+
Valine, Leucine, Isoleucine	0.94(d);0.96(d);1.0(d); 1.01(d);1.05(d)	-			
Lysine	1.72(m);2.98(m)	-			
Glutathione	2.17(m);2.56(m); 2.94(m)			+	
Glycogen	5.42(m)			-	
Aspartic acid	2.65(dd);2.80(dd)				+
GABA	1.90(q);2.27(t);3.01(t)				-

"+" represents significantly elevated concentration and "-" represents significantly lowered concentration. Chemical shifts (ppm) are relative to TMSP (^{1}H, δ). Multiplicity of signals is indicated in brackets: s= singlet, d= doublet, dd= doublet of doublet, t= triplet, q= quadruplet and m= multiplet.

[a] LDL and VLDL

[b] phosphatidyl-choline and glycero-phosphocholine

Table 2. Pairwise model parameter comparison of PND2 whole body and PND21 liver, brain and serum.

Groups	N	PLS components	$R^2Y(\%)$	Q^2 cum
PND2 Whole body - Control/BPA 0.025µg	38	2	99.3	0.979
PND2 Whole body - Control/BPA 0.25µg	34	2	99.4	0.970
PND2 Whole body - Control/BPA 25µg	31	1	99.5	0.989
PND2 Whole body - BPA 0.025µg/BPA 0.25µg	31	3	98.0	0.731
PND2 Whole body - BPA 0.025µg/BPA 25µg	29	1	98.3	0.943
PND2 Whole body - BPA 0.25µg/BPA 25µg	25	2	98.9	0.969
PND21 Liver - Control/BPA 0.025µg	21	1	89.7	0.822
PND21 Liver - Control/BPA 0.25µg	24	1	99.5	0.980
PND21 Liver - Control/BPA 25µg	24	2	99.7	0.990
PND21 Liver - BPA 0.025µg/BPA 0.25µg	23	3	99.5	0.896
PND21 Liver - BPA 0.025µg/BPA 25µg	24	3	99.4	0.950
PND21 Liver - BPA 0.25µg/BPA 25µg	26	2	98.8	0.928
PND21 Brain - Control/BPA 0.025µg	22	2	99.0	0.955
PND21 Brain - Control/BPA 0.25µg	22	2	99.0	0.963
PND21 Brain - Control/BPA 25µg	21	1	90.0	0.664
PND21 Brain - BPA 0.025µg/BPA 0.25µg	23	3	98.5	0.932
PND21 Brain - BPA 0.025µg/BPA 25µg	25	3	99.5	0.941
PND21 Brain - BPA 0.25µg/BPA 25µg	26	4	99.4	0.895
PND21 Serum - Control/BPA 0.025µg	23	3	99.6	0.826

Groups	N	PLS components	$R^2Y(\%)$	Q^2 cum
PND21 Serum - Control/BPA 0.25µg	25	4	98.9	0.932
PND21 Serum - Control/BPA 25µg	23	3	99.8	0.991
PND21 Serum - BPA 0.025µg/BPA 0.25µg	26	3	96.3	0.891
PND21 Serum - BPA 0.025µg/BPA 25µg	23	2	99.0	0.937
PND21 Serum - BPA 0.25µg/BPA 25µg	26	3	99.3	0.973

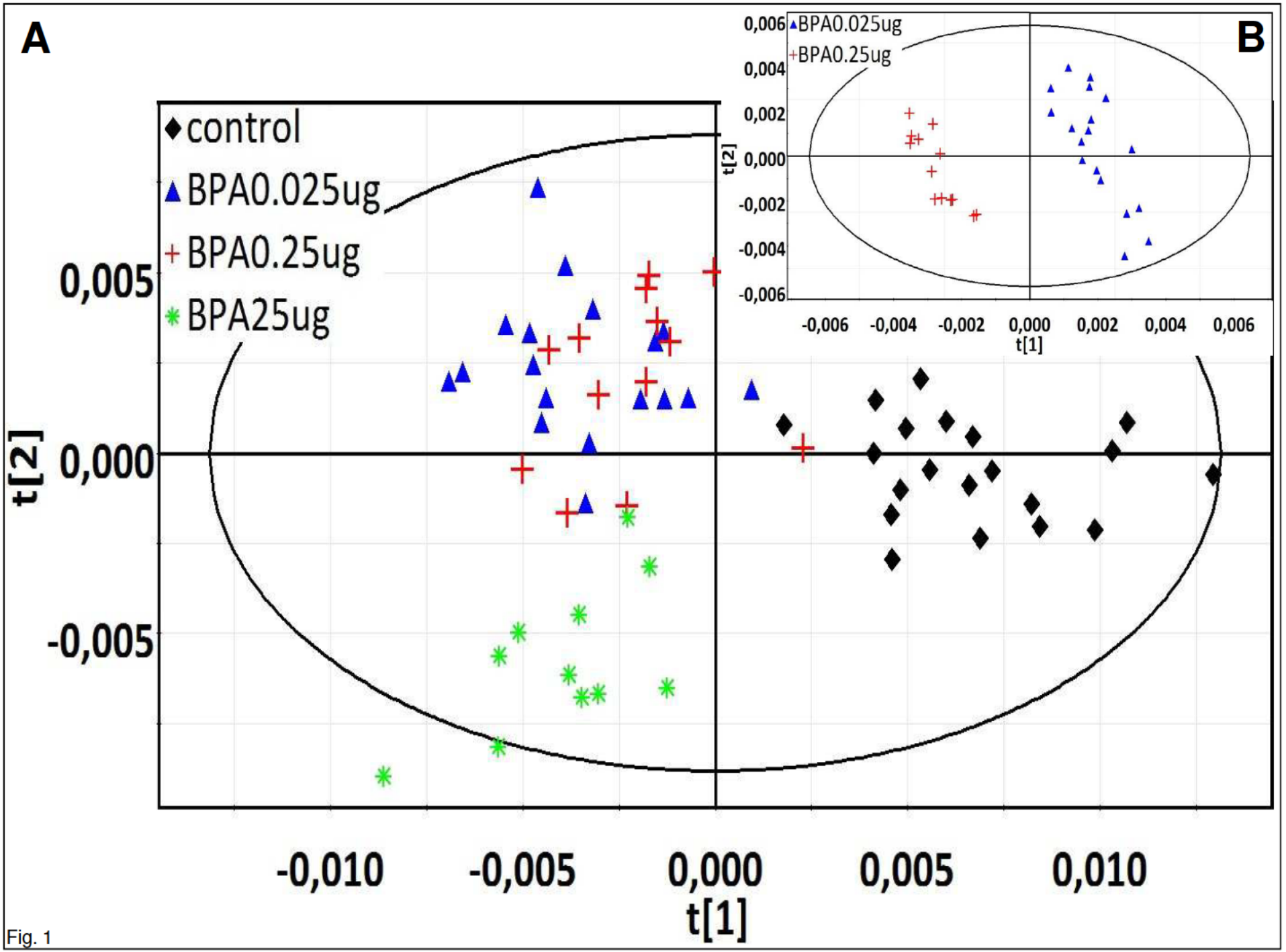
Figure Legends

Figure 1. Two-dimensional PLS-DA scores plot of PND2 whole body F1 male extracts integrated ^{1}HNMR spectra. (A) Control (N=20), BPA0.025µg/kg (N=18), BPA0.25µg/kg (N=14), BPA25µg/kg (N=11) (1st and 2nd latent variable out of 3 components: R^2Y=71.5% and Q^2=0.557). (B**)** BPA0.025µg and BPA0.25µg taken separately (1st and 2nd latent variable out of 3 components: R^2Y=73.1% and Q^2=0.731).

Figure 2. Two-dimensional PLS-DA score plot of PND21 serum F1 male integrated ^{1}HNMR spectra. (A) Control (N=11), BPA0.025µg (N=12), BPA0.25µg (N=14) and BPA25µg (N=12) (2 components): R^2Y=55.3% and Q^2=0.450. **(**B**)** Control and BPA0.025µg. (3 components: R^2Y=99.6% and Q^2=0.826) (C) Control and BPA0.25µg. (4 components: R^2Y=98.9% and Q^2=0.932)

Figure 3. Two-dimensional PLS-DA scores plot of PND21 liver F1 male extracts integrated ^{1}HNMR spectra. (A) Control (N=11), BPA0.025µg (N=11), BPA0.25µg (N=13), BPA25µg/kg (N=14) (1st and 2nd latent variable out of 3 components: R^2Y 48.3% and Q^2=0.421). (B) Control and BPA0.025µg. (1 component: R^2Y=89.7% and Q^2=0.822) (C) BPA0.025µg and BPA0.25µg. (3 components: R^2Y=99.5% and Q^2=0.896) (D): BPA0.025µg and BPA25µg. (3 components: R^2Y=99.4% and Q^2=0.950)

Figure 4: Two-dimensional PLS-DA scores plot of PND21 brain F1 male extracts integrated ^{1}H-NMR spectra for Control (N=11), BPA0.025µg (N=11), BPA0.25µg (N=13), BPA25µg/kg (N=14) (1st and 2nd latent variable out of 3 components: R^2Y=78.9% and Q^2=0.564).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BPA0.25ug
BPA25ug
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0,000
-0,005
t[2]
-0,010
-0,005
0,000
0,005
0,010
t[1]
B
BPA0.025ug
BPA0.25ug
0,006
0,004
0,002
0,000
-0,002
-0,004
-0,006
t[2]
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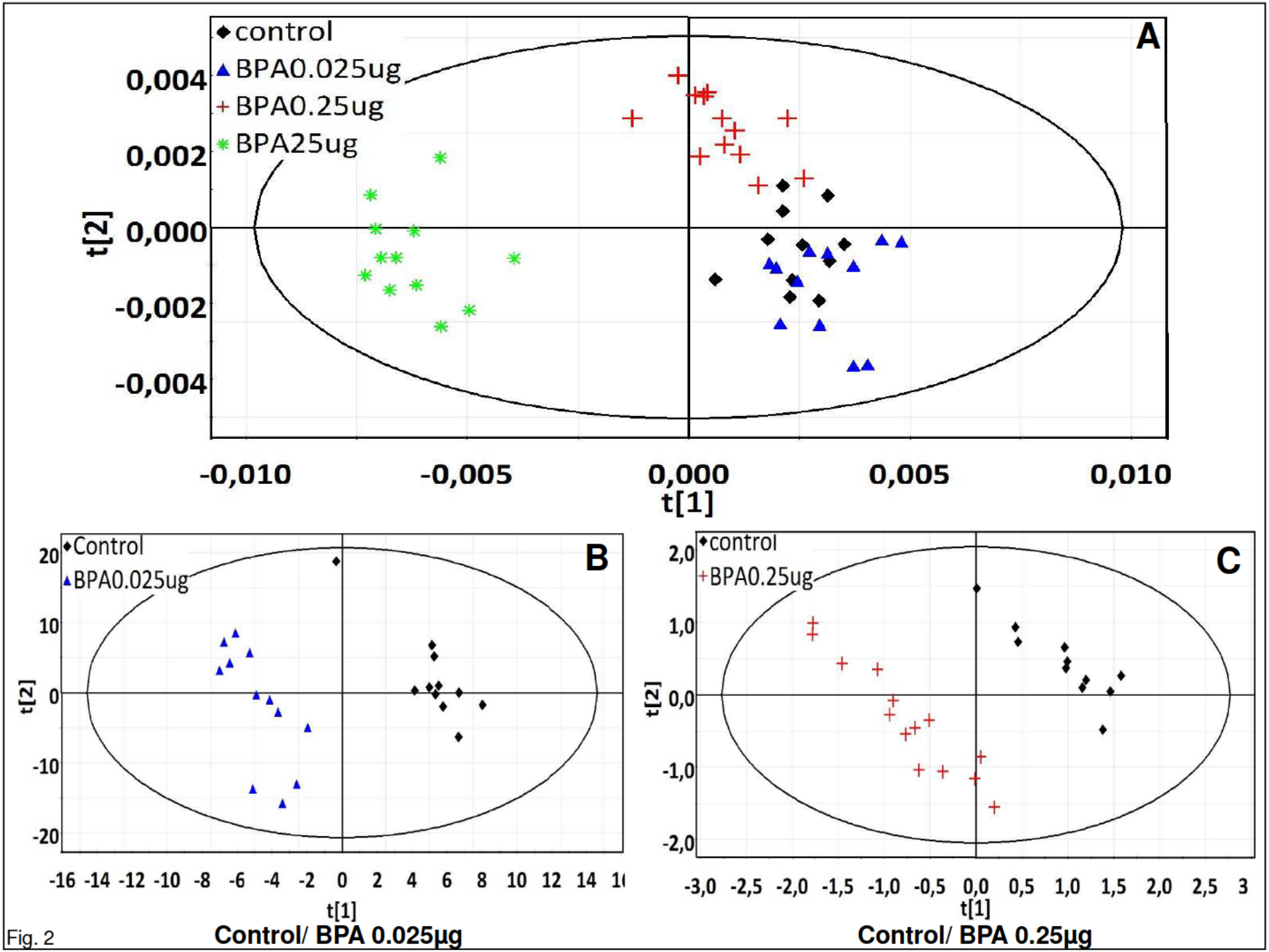
-0,004
-0,002
0,000
0,002
0,004
0,006
t[1]

Fig. 1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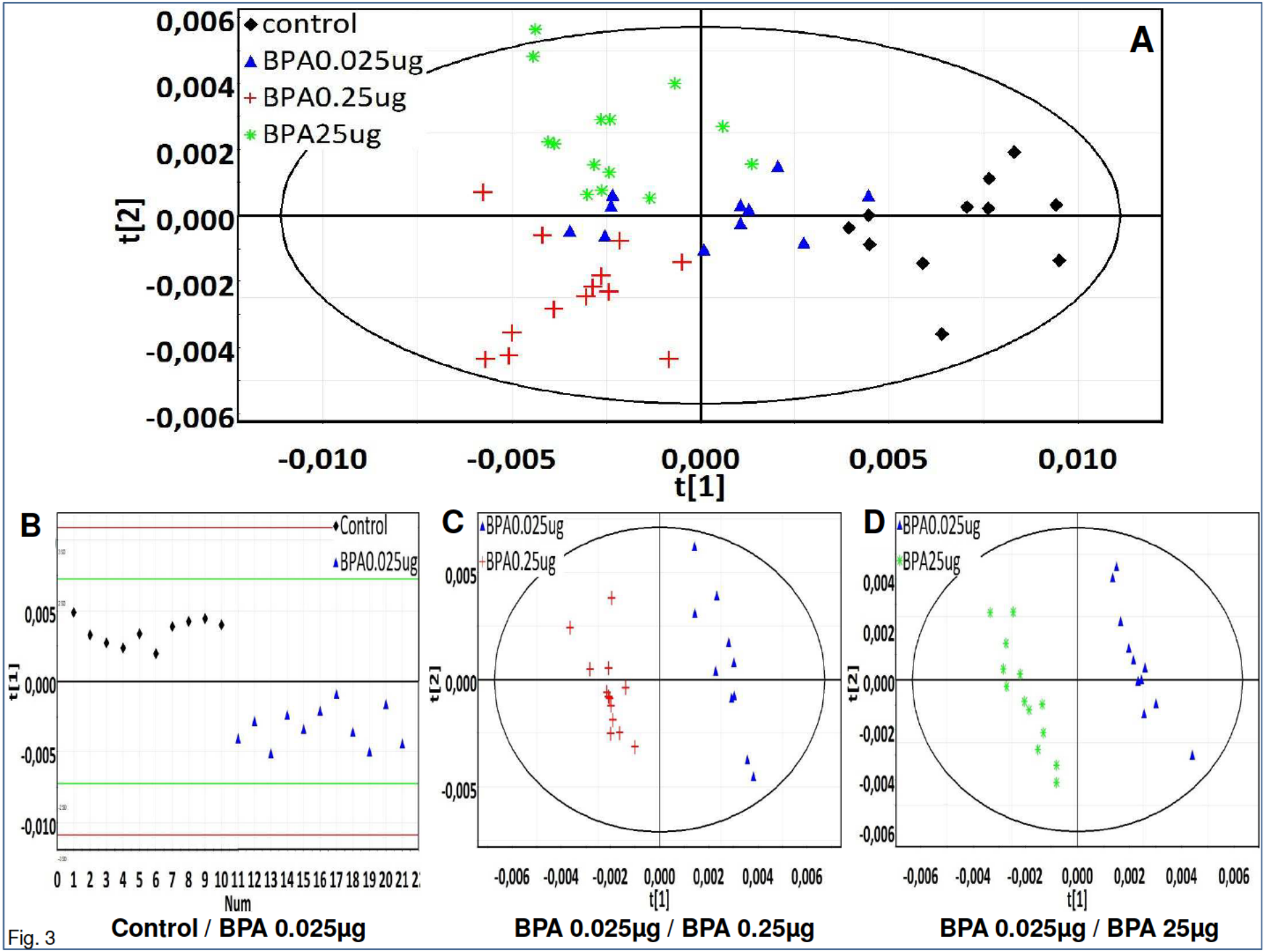
-0,002
-0,004
t[2]
-0,010
-0,005
0,000
0,005
0,010
t[1]
Control
BPA0.025ug
B
20
10
0
-10
-20
t[2]
-16 -14 -12 -10 -8 -6 -4 -2 0 2 4 6 8 10 12 14
t[1]
Control/ BPA 0.025µg
control
BPA0.25ug
C
2,0
1,0
0,0
-1,0
-2,0
t[2]
-3,0 -2,5 -2,0 -1,5 -1,0 -0,5 0,0 0,5 1,0 1,5 2,0 2,5 3
t[1]
Control/ BPA 0.25µg

Fig. 2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t[1]
Num
Control / BPA 0.025µg
C
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BPA0.25ug
t[2]
t[1]
BPA 0.025µg / BPA 0.25µg
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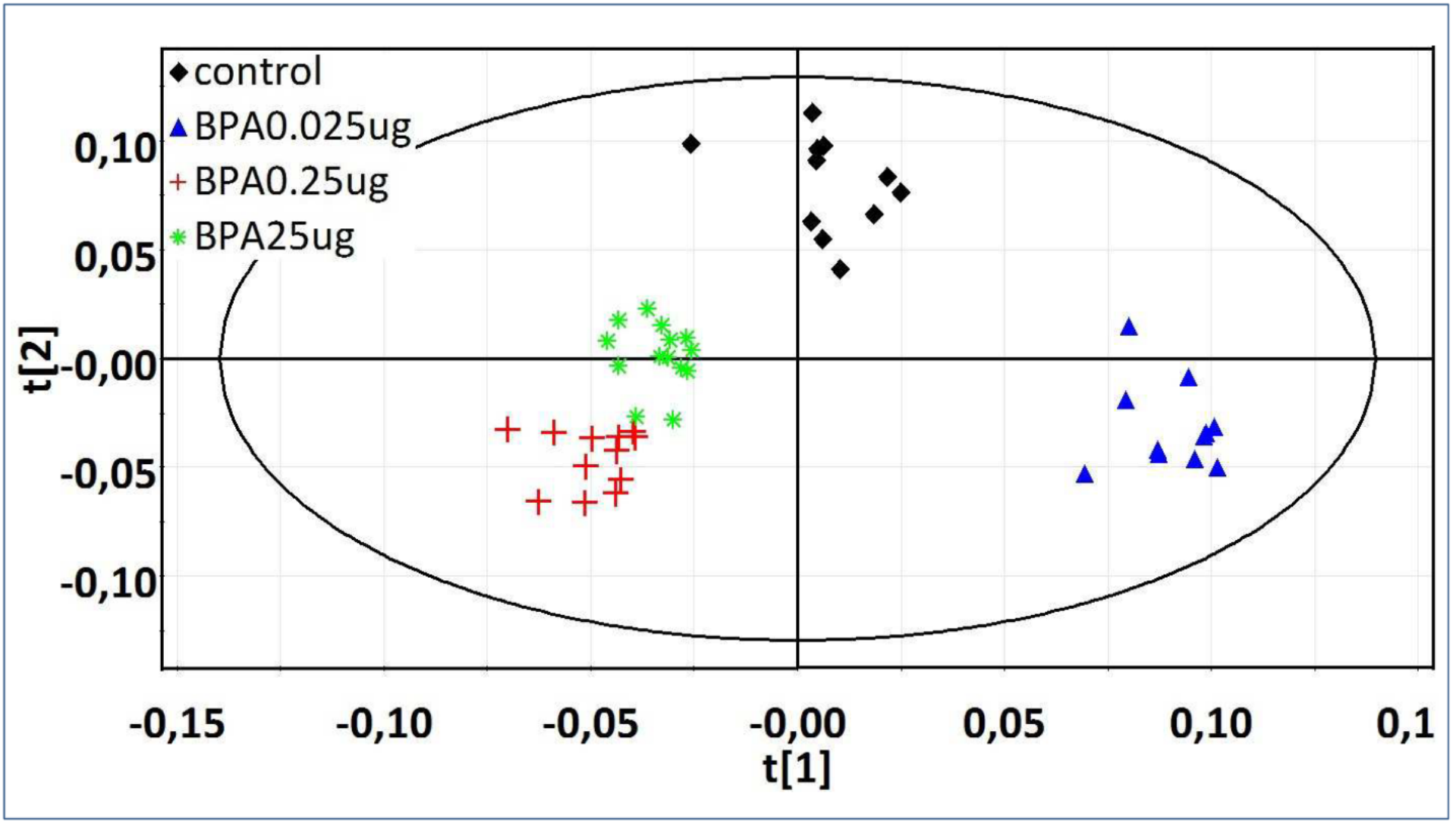
t[2]
t[1]
BPA 0.025µg / BPA 25µg

Fig. 3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t[1]

Fig 4